Original Article

Kin17 knockdown suppresses the migration and invasion of cervical cancer cells through NF-κB-Snail pathway

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Abstract: Cervical cancer is one of the most common cancers in women worldwide. Metastasis in cancer has been a Gordian knot due to unsatisfactory clinical treatments. KIN17, a highly conserved gene from yeast to human, up-regulation is associated with the pathogenesis and development of several common cancers. Our previous works revealed that elevated expression of kin17 observed in cervical cancer tissues showed a close association with lymph node metastasis. This study aimed to explore roles and mechanisms of kin17 in the migration and invasion of cervical cancer cells. Cervical cancer cell lines HeLa and SiHa with kin17 knockdown were constructed by using recombinant lentiviral vector that carry specific siRNA targeting KIN17 gene. The mRNA and protein levels of kin17 in cells were determined by RT-qPCR and western blotting, respectively. Wound healing assay and transwell assays were performed to assess the migration and invasion abilities of the cancer cells, respectively. The expression of signaling proteins involved in the NF-κB-Snail pathway was analyzed by western blotting. As our results showed, the mRNA and protein levels of kin17 in cells were determined by RT-qPCR and western blotting, respectively. Wound healing assay and transwell assays were performed to assess the migration and invasion abilities of the cancer cells, respectively. The expression of signaling proteins involved in the NF-κB-Snail pathway was analyzed by western blotting. As our results showed, the mRNA and protein levels of kin17 in HeLa cells and SiHa cells showed a significant decrease by transfection with recombinant lentiviral vector carrying specific siRNA. Compared with control group, the migration rates were decreased in the kin17 knockdown group in both HeLa and SiHa cells in wound healing assay as well as transwell assay without matrigel. Kin17 knockdown also reduced the cell invasion number of both HeLa and SiHa cells. In addition, the phosphorylation of nuclear factor Kappa B (NF-κB) p65, IKappa B kinase α (IKKα), and IKappa B α (IκBα) in NF-κB pathway and the expression of Snail were decreased in HeLa cells and SiHa cells by kin17 knockdown. Our results demonstrated that knockdown of kin17 in cervical cancer cells suppressed cell migration and invasion, and inhibited the activity of NF-κB signaling pathway and the expression of Snail. These findings suggested kin17 as an essential regulator of the cell migration and invasion and the underlying molecular mechanism involved NF-κB-Snail pathway in cervical cancer. This might serve as a novel molecular therapeutic target for treating cervical cancer metastasis.

Keywords: Kin17, migration, invasion, cervical cancer, NF-κB-Snail pathway

Introduction

Cervical cancer is one of the most malignancies in women worldwide, and is still posing a serious threat to women, showing high incidence in the younger generations [1-4]. Although ultrasonography, cervical smear cytological examination and human papillomavirus (HPV) genotyping are routinely used for clinical screening of cervical cancer, many patients are often detected in advanced stages [5]. Moreover, the treatment outcomes of cancer metastasis still remained unsatisfactory. Thus, it is necessary to elucidate the molecular mechanisms for cancer metastasis in order to develop new therapeutic targets for cervical cancer.
Kin17 is a highly conserved gene from yeast to humans, and encodes a protein kin17 with a molecular weight of 45 KDa. According to previous studies, kin17 has been reported to participate in DNA replication [6], DNA damage response [7] and cell cycle progression [8]. Recently, kin17 has been found to be up-regulated in several common cancers including breast cancer [9], colorectal cancer [10], and lung cancer [11], and is related to the pathogenesis and development of these cancers. Our previous study demonstrated that kin17 played an important role in the invasion and metastasis of non-small cells lung cancer (NSCLC) [11]. Elevated expression of kin17 is also observed in cervical cancer samples, showing a close association with lymph node metastasis [12]. However, the association of kin17 with metastasis of cervical cancer remained unclear. Therefore, this study aimed to explore the roles and the relevant mechanisms of kin17 in the migration and invasion of cervical cancer cells in this study.

Materials and methods

Cell culture

Human cervical cancer cell lines HeLa and SiHa were obtained from GeneChem Company (Shanghai, China) and were cultured in Dulbecco Modified Eagle Medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Tianjin Kangyuan, Biotechnology Co., Ltd.), 60 μg/mL penicillin and 100 μg/mL streptomycin (Hyclone, USA). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_{2}.

Lentiviral vector construction and cell lines screening

The gene-silencing lentiviral vector GV248-KD with specific siRNA targeting KIN17 gene sequence and the normal controlled lentiviral vector GV248-NC were successfully constructed, as described previously [11]. Lentiviral vector GV248-KD contained a reporter gene enhanced green fluorescent protein (EGFP). After gene transfection, virus particles transfected with gene-silencing lentiviral vector (HeLa^{KD} cells) or the controlled vector (SiHa^{NC} cells), were cultured with puromycin until the cells reach ~90% confluence with positive EGFP expression. The cells with stable transfection were maintained in Dulbecco’s Modified Eagle Medium supplemented with 10% FBS and puromycin. HeLa cells or SiHa cells without transfection with vector (HeLa^{Mock} cells or SiHa^{Mock} cells) were used as blank control.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the cells and was prepared for cDNAs synthesis using a reverse transcription kit (Promega Corporation, Madison, WI, USA). The primers for KIN17 gene were as follows: forward: 5'-CCATGATCTTCTCATTTGC-3', reverse: 5'-GTAATACGTTATCCACGGG-3'. The primers for GAPDH were as follows: forward, 5'-GGAGCGAGATCCCTCAAAT-3'; reverse, 5'-GGCTGTTGTTCATACCTCTCATGG-3'. The cDNA was then used for PCR amplification with SYBR Premix Ex Taq (cat. no., DRR420A, TaKaRa Bio, Inc., Otsu, Japan) in a thermal cycler (GeneAmp 2400; PE Applied Biosystems, Foster City, CA, USA). All samples were run in triplicate and the relative mRNA levels were calculated using the 2^{-ΔΔCq} method provided by the System software (Applied Biosystems).

Western blot analysis

Total proteins from the cells were prepared for western blot analysis. RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) containing a complete protease inhibitor cocktail tablet (Roche Applied Science, Penzberg, Germany) was used to extract the total proteins as described previously [9]. A 12% SDS-PAGE were used to separate 100 μg of protein per lane and then transferred onto Immobilon®-P PVDF Transfer Membranes (EMD Millipore, Billerica, MA, USA). Next, the protein sample was blocked with non-fat milk at room temperature for 1 hour, and the membranes were incubated with monoclonal primary antibodies using anti-kin17 (dilution, 1:500; cat. no. sc-32769; Santa Cruz Biotechnology, Inc.), anti-Snail (dilution, 1:1000; cat. no. #3879; Cell Signaling TECHNOLOGY, Inc.), anti-IKKα (dilution, 1:1000; cat. no. #11930; Cell Signaling TECHNOLOGY, Inc.), anti-NF-κB p65
Association of kin17 and cervical cancer

(dilution, 1:1000; cat. no. #8242; Cell Signaling TECHNOLOGY, Inc.), anti-IκBα (dilution, 1:1000; cat. no. #4814; Cell Signaling TECHNOLOGY, Inc.), anti-phospho-IKKα (dilution, 1:1000; cat. no. #2697; Cell Signaling TECHNOLOGY, Inc.), anti-phospho-NF-κB p65 (dilution, 1:1000; cat. no. #3033; Cell Signaling TECHNOLOGY, Inc.), anti-phospho-IκBα (dilution, 1:1000; cat. no. #2859; Cell Signaling TECHNOLOGY, Inc.) and anti-GAPDH (dilution, 1:25000; cat. no. 60004-1-ig; proteintech, Inc.) for overnight at 4°C. After washing with TBST, HRP-conjugated secondary antibodies (dilution, 1:1000; cat. no. #7074/7076; Cell Signaling TECHNOLOGY, Inc.) were used for developing immunoblots at room temperature for 1 hour, and were processed using ECL enhanced chemiluminescence substrate (Thermo Fisher Scientific, Inc.). Images were captured using the ImageQuant RT ECL™ imager (GE Healthcare Life Sciences, Shanghai, China). Band intensities were quantified by using ImageJ software (GE Healthcare Life Sciences, Shanghai, China).

Wound healing assay

Cells were seeded into 6-well plates (3×10^5 cells/well). Next, a sterile 200 μL micropipette tip was used to make the wound when the cells reached to 90%. The wounded monolayers were washed with phosphate buffer solution (PBS, Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) three times to remove the cell debris. The gap between the two edges of the wound was measured after incubation at 0, 24 and 48 hours.

Transwell assay without matrigel

Transwell assay without matrigel was performed to evaluate the migration ability of HeLa and SiHa cells [13]. A cell density of 5×10^4 cells were seeded in serum-free medium at a volume of 200 μL/well in the upper chamber. The lower chambers were filled with 600 μL medium supplemented with 30% FBS as chemoattractants. After incubation for 24 hours, the cells in the upper chambers were removed by wet cotton swabs. Migrated cells on the lower side of the filter were fixed with 100% methanol (Guangdong Guanghua Sci-Tech Co., Ltd.) and stained with 0.25% crystal violet solution (Shanghai GeneChem Co.) for 30 minutes. After washing with PBS for three times, the migrated cells in the lower chamber were observed and then photographed using an inverted microscope. The numbers of migrated cells was determined by photographing in five random fields per chamber at 200× magnification.

Transwell assay with matrigel

Transwell assay with matrigel was performed to evaluate the invasive capacity of the cancer cells [14]. Briefly, transwell chambers (Costar®, Corning Incorporated, Corning, NY, USA) with 8 μm pore polycarbonate filters were coated with Matrigel (BD Biosciences, San Jose, CA, USA) at 37°C for 1 hour for solidification. A cell density of 5×10^4 cells were seeded in the upper chamber at a volume of 200 μL and incubated for 48 hours. Invasive cells were stained and counted, which followed a protocol that was similar to transwell assay without matrigel.

Statistical analysis

All experiments were repeated three times and the data were presented as mean ± SD. SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. All statistical results and corresponding p values reported were two-tailed. P<0.05 was considered to be statistically significant difference.

Results

Cervical cells lines HeLa and SiHa with kin17 knockdown were established

To investigate the function of kin17 in cervical cancer cells, the recombinant lentiviral vector with or without KIN17 siRNA were used to transfect HeLa and SiHa cells and then the stable expression cell colonies were screened by puromycin for nearly a month. The results of RT-qPCR assay revealed that the mRNA levels of Kin17 in HeLaKD cells (Figure 1C) and SiHaKD cells (Figure 1D) were significantly decreased when compared with levels in HeLaNC cells or SiHaNC cells (P<0.05, n=3), respectively. Furthermore, western blot analy-
Association of kin17 and cervical cancer

sis confirmed that the protein levels of kin17 in HeLaKD cells (Figure 1E) and SiHaKD cells (Figure 1F) showed a significant decrease when compared with those in the controlled cells (P<0.05, n=3), respectively.

Kin17 knockdown suppressed the migration and invasion of HeLa and SiHa cells

At 24 or 48 hours following the scratch, wound healing assay showed that the rates of migration in HeLaKD cells (P<0.05, n=3, Figure 2A) and SiHaKD cells (P<0.05, n=3, Figure 2B) showed a significant decrease when compared with those in HeLaNC cells or SiHaNC cells, respectively. Furthermore, transwell assay without matrigel was used to determine the migration ability of cells. In consistent with the results of wound healing, the migrated cells were also significantly decreased in both HeLaKD cells (P<0.05, n=3, Figure 2C) and SiHaKD cells (P<0.05, n=3, Figure 2D) compared with the controlled groups.

In transwell assay with matrigel, an experiment that was performed for testing the invasive ability of cells, the numbers of invaded cells showed a significant reduction in HeLaKD cells with those in HeLaNC cells or SiHaNC cells, respectively.
Association of kin17 and cervical cancer

(P<0.05, n=3, Figure 3A) and SiHa\textsuperscript{KD} cells (P<0.05, n=3, Figure 3B) when compared to control groups.

**Kin17 knockdown changed expression of the signaling molecules of NF-κB-Snail pathway in HeLa and SiHa cells**

To explore the downstream signaling molecules of kin17 in cervical cancer cells, the expression of the important proteins that are involved in NF-κB signaling pathway and epithelial-mesenchymal transition (EMT) were compared by western blotting. Our results showed that phosphorylation of NF-κB p65, IKKα and IκBα in NF-κB pathway showed a decrease in HeLa\textsuperscript{KD} cells and SiHa\textsuperscript{KD} cells (Figure 4A and 4B). The expression of transcriptional factor Snail in HeLa\textsuperscript{KD} cells and SiHa\textsuperscript{KD} cells was lower than that in HeLa\textsuperscript{NC} cells and SiHa\textsuperscript{NC} cells (Figure 4C and 4D), respectively.

*Figure 2.* Effect of kin17 knockdown on migration ability of cervical cancer cells. Representative images and quantification of HeLa (A) and SiHa (B) cells in wound healing assay, ×100. Representative images and quantification of HeLa (C) and SiHa (D) cells in transwell assay without matrigel, ×200; *P<0.05, n=3.*
Discussion

Several studies have reported that kin17 was involved in the metastasis of several types of cancers. Our previous studies have revealed that up-regulation of kin17 was found in clinical specimens of cervical cancer, and it was associated with lower tumor differentiation, and high risk of lymph node metastasis in a retrospective analysis [11]. To investigate the role of kin17 in the metastasis of cervical cancer, two cell lines with kin17 knockdown by using lentiviral vector transfection were successfully constructed, and the levels of mRNA and protein using RT-qPCR and western blotting, respectively were performed. The results revealed that kin17 knockdown inhibited migration of both HeLa and SiHa cells and suppressed invasion of the two cell lines.

The underlying molecular mechanisms of kin17 in the migration and invasion of cervical cancer cells were further investigated in this study. NF-κB pathway has been identified as one of the key signaling pathways in the metastasis of cancer [15-18]. Activation of NF-κB, a nuclear transcription factor, regulates the expression of downstream oncogenes. Our study revealed that kin17 knockdown in cervical cancer cells decreased the phosphorylation of NF-κB p65, IKKα and IkBα, which are three important components of NF-κB signaling pathway. This suggested that kin17 was elevated in cervical cancer tissues, which contributed to the activation of NF-κB pathway and progression of the cancer. Additionally, NF-κB-Snail pathway in cancer cells showed an association with EMT, and closely related to the stemness, metastasis, and progression in most of the cancers [19-21], including cervical cancer [22]. EMT and phenotype of cancer cells are controlled by the gene regulatory network. As a downstream of NF-κB signaling in NF-κB-Snail pathway, transcriptional factor Snail was involved in the EMT process, contributing to the migration, invasion and even distant metastases of cancer cells through blocking E-cadherin gene and stimulating N-cadherin gene [23, 24]. We found that the phosphorylation of NF-κB signaling factors and the expression of Snail were decreased after kin17 knockdown. This indicated that kin17 might regulate the migration and invasion abilities of cervical cancer cells through NF-κB-Snail pathway, which has been identified as one of the most important signaling pathways for cancer cell metastasis [25, 26].

In conclusion, knockdown of kin17 which was up-regulated in cervical cancer tissues; and inhibited cervical cancer cell migration and invasion via NF-κB-Snail pathway. Kin17 might
Association of kin17 and cervical cancer

**A**

HeLa

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**C**

HeLa

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**D**

SiHa

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act as a novel molecular therapeutic target for the metastasis of cervical cancer. However, further experiments are required to understand the roles, molecular mechanisms and potential clinical application of kin17 in the metastasis of cervical cancer cells.

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Disclosure of conflict of interest

None.

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References


Figure 4. Effect of Kin17 knockdown on the expression of signaling molecules of NF-κB-Snail pathway in cervical cancer cells. Phosphorylation of IKKα, IkBα, NF-κB p65 (A and B) and expression of Snail (C and D) after kin17 knockdown in HeLa and SiHa cells were detected through western blotting analysis. IKKα, Ikappa B kinase α; IkBα, Ikappa B alpha; NF-κB, nuclear factor kappa B.
through inhibition of NF-Kappa B-induced endothelial activation. FASEB J 2015; 29: 227-38.


